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Full-length nucleotide sequences of *mcr-1* harboring plasmids isolated from extended-spectrum β-lactamase (ESBL)- producing *Escherichia coli* of different origins

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Abstract

Here we present the full sequences of three *mcr-1* carrying plasmids isolated from ESBL-producing *E. coli*. The plasmids belong to three different replicon types and are 34.640 bp, 209.401 bp and 247.885 bp in size, respectively. We describe for the first time a composite transposon containing *mcr-1* localized on an MDR-IncHI2 plasmid harboring additional determinants of resistance to six different classes of antibiotics including the ESBL-gene $bla_{\text{CTX-M-1}}$ and heavy metal resistance.

The recent description of the plasmid-mediated colistin resistance gene, *mcr-1*, in strains isolated from food animals, food and humans in China was a trigger for an avalanche of retrospective studies investigating the presence of this specific gene (1). Since then, *mcr-1* has been found almost all over the world and the earliest evidence for its presence dates back to the 1980's (2). The wide spread of *mcr-1* and the finding that this resistance marker is often associated with multidrug resistant *Enterobacteriaceae*, e.g. extended-spectrum β-lactamase (ESBL)-producers or carbapenemase-producers, is of great concern to public health (3).

The *mcr-1* gene was so far associated with different plasmid replicon types such as IncI2, IncHI2, IncP, IncFIP and IncX4 (1,4,5,6,7). However, only very limited data about the complete sequences of such plasmids and the genetic structure surrounding the *mcr-1* gene are available. In this study, we present the complete sequence of three distinct *mcr-1* harboring plasmids isolated from ESBL-producing *E. coli* isolates originating from river water (*E. coli* OW3E1, B1:ST359; SHV-12), imported vegetables from Thailand (*E. coli* H226B, A:ST167, CTX-M-55) and imported poultry meat from Italy (*E. coli* S38, B1:ST602, CTX-M-1) (8, 9).

The *mcr-1* harboring plasmids were transferred by transformation experiments into *E. coli* DH5-alpha and colistin resistant transformants were selected on LB-agar supplemented with 2 mg/L colistin (SIGMA, St. Louis, USA). The *mcr-1* harboring plasmids pOW3E1, pH226B and pS38 were extracted using the Large-Construct Kit (Qiagen, Hombrechtikon, Switzerland) according the manufacturer's protocol. The plasmids were sequenced on a PacBio RS2 device (Pacific Biosciences, Menlo Park, USA) with a 10 kb size-selected insert library and P6/C4 chemistry. pOW3E1 and pH226B were multiplexed with four other plasmids (data not shown) in a barcoded library (symmetric 384 barcode set) and run on two SMRT cells; pS38 was prepared as single library and sequenced on another SMRT cell.

De-Barcoding, whitelisting and *de novo* assembly (HGAP3 algorithm) was performed using SMRTanalysis version 2.3 (Pacific Biosciences). HGAP3 settings were kept at the defaults, except for the expected genome size, which was set between 60 and 200. The HGAP3 analysis produced complete plasmid sequences of the multiplexed samples with at least 50-fold coverage over the entire molecules. Plasmid pS38 was sequenced individually with 2250-fold coverage. The plasmid sequence was automatically annotated using the online Rapid Annotation Subsequencing Technology (RAST) and CLC Main Workbench Version 7.7 (CLC bio, Aarhus, Denmark) (10). Automated annotation was manually refined using the BLASTn and BLASTp programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Plasmid pOW3E1 is 34,640 bp in size, with a G+C content of 42.3% (Table 1). The plasmid backbone is similar to that of other IncX4 plasmids including a type IV secretion system (pilX operon), which is associated with this plasmid group (data not shown). Interestingly, no resistance determinants other than the *mcr-1* gene were located on pOW3E1.

Plasmid pH226B is 209,401 bp in size with a G+C content of 46.5% (Table 1). It consists of a typical IncHI1 backbone and shares many open reading frames (orf) with the reference plasmid of the IncHI1 group R27 (GenBank accession number AF250878). Beside the *mcr-1* gene, a Tn3 family transposon carrying heavy metal resistance determinants (e.g. Zn^{2+}, Cu^{2+} or Ag^{2+}) is present on pH226B as well as a remotely located *tcuABC* operon that enables the use of tricarballylate as carbon/energy source.

Plasmid pS38, extracted from an *E. coli* isolate originating from imported poultry meat, represents a large multi-drug-resistant (MDR) plasmid (247,885 bp, G+C content: 46.7%) with high similarity in its backbone to the IncHI2 reference plasmid R478 (accession no. NC_005211) (Table 1). The MDR-region of this plasmid includes two class 1 integrons carrying transposons In641 (*estX-3, psp, aadA2, cmlA1, aadA1a, qacH2*) and In369 (*dfrA1b, aadA1b*), conferring resistance to aminoglycosides, chloramphenicol, quaternary ammonium compounds and

trimethoprim. Between these transposons, the macrolide efflux pump gene *mefB*, the ESBL-gene $bla_{\text{CTX-M-1}}$ and the sulphonamide resistance gene *sul3* are present. Downstream of transposon In369, two transposons carrying mercury resistance determinants and tetracycline resistance determinants, respectively, followed. Furthermore, a tellurite resistance operon is present on pS38.

The *mcr-1* genes were found to be located within various genetic contexts in the sequenced plasmids (Figure 1). In all three plasmids, a 735 bp orf encoding a hypothetical protein with similarities to a PAP2 superfamily protein was detected immediately downstream of the *mcr-1* gene (both together hereafter referred to as *mcr-1* element).

In pOW3E1 the *mcr-1* gene is inserted approximately 1.5 kb upstream of the replicon initiation protein and is found close to an IS*2*-like insertion element. An IS*Apl1* element is absent. In pH226B, the *mcr-1* element is embedded in the IncHI1 backbone between two genes that were highly similar to those annotated as Orf061 and Orf062 on the IncHI1 reference plasmid R27. No mobile element was present upstream or downstream nearby. In pS38, the *mcr-1* element is flanked by two IS*Apl1* elements (composite transposon) and is located directly upstream of the MDR-region.

To our knowledge, this is the first description of an MDR-IncHI2 plasmid harboring a composite transposon containing the *mcr-1* gene and determinants for resistance to six different classes of antibiotics including the ESBL-gene $bla_{\text{CTX-M-1}}$ and additional heavy metal resistance determinants. The fact that the *mcr-1* gene is found to be part of a composite transposon is highly worrisome, since accelerated dissemination by illegitimate recombination of the whole transposon to either plasmids or chromosoms is likely.

Furthermore, we present for the first time an IncHI1 plasmid carrying the mobile colistin resistance determinant. The presence of heavy metal resistance determinants on pS38 and

pH226B might lead to co-selection, for example by zinc which is widely used as a feed additive for fattening pigs.

Since the *mcr-1* element was found on different plasmid types and the IS*Apl1* element was not always present we hypothesize that the *mcr-1* gene may have been mobilized independently several times. In the case of pH226B however, where the *mcr-1* gene is embedded in the middle of the backbone without any evidence for the presence of mobile elements, no conclusions concerning the mobilization of this gene can be made.

Nucleotide sequence

The GenBank accession numbers for pS38, pOW3E1 and pH226B are KX129782, KX129783, KX129784, respectively.

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Fig 1. The genetic structures surrounding the *mcr-1* gene in plasmids of this study compared to plasmid pHNSHP45. The arrows indicate open reading frames (orf), with black, stippled, grey and white arrows representing *mcr-1,* IS elements, orf with known function and orf with unknown function, respectively.